Consequences of strain variability and calcification in *Emiliania huxleyi* on microzooplankton grazing

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ABSTRACT

Microzooplankton are the main consumers of marine phytoplankton. Intrinsic traits of phytoplankton can reduce grazing mortality, directly influencing phytoplankton population dynamics, food web ecology, and biogeochemical cycling. We examined the impact of calcification in mediating the functional grazing response of three heterotrophic dinoflagellates, on the coccolithophore, *Emilania huxleyi*. A variety of parameters, including predator grazing and growth rates, were examined over a 24-48 h period, at 1-5 prey concentrations for five isolates of *E. huxleyi* that fell along a gradient of calcification states. Significant differences in ingestion and clearance rate were strain-specific, and no apparent trends were observed in relation to calcification. However, predators had, on average, a had a 60% slower growth rate on calcified strains relative to naked strains; furthermore, gross growth efficiency was reduced when ingesting calcified strains. A growth rate model demonstrated a positive feedback from grazing interactions whereby decreased predator growth rate on calcified strains resulted in the accumulation of *E. huxleyi*. This study highlights the complexity involved in understanding the role of prey phenotype on grazing rates, and emphasizes the importance in considering morphological traits when deciphering predator-prey interactions in the plankton.

INTRODUCTION

The coccolithophore, *Emiliania huxleyi* is a globally important phytoplankter that can form massive annual blooms that extend hundreds of kilometers, and are observable from space. The alga is a central component of the global carbon cycle, as their calcite coccoliths account for a third of total marine $CaCO_3$ production (Balch et al. 1992; Iglesias-Rodriguez et al. 2008). Further, *E. huxleyi* produces the osmolyte dimethylsulphoniopropionate (DMSP), which, once excreted, can be cleaved to produce dimethlysulfide (DMS), the predominant source of atmospheric sulfur and important in cloud formation (Simó 2001). Thus, understanding factors mediating population abundance and distribution of this algal species is important for predicting its role in marine biogeochemical cycling and global climate.

Phytoplankton population dynamics are driven by the relative rates of cell growth and loss. *E. huxleyi* typically blooms under highly stratified conditions (Nanninga and Tyrrell 1996), and its high light saturation irradiance (Balch et al. 1992; Nielsen 1995; Nanninga and Tyrrell 1996) and tolerance of low nutrient conditions (Brand 1991; Muggli and Harrison 1997; Paasche 1998, Riegmann 2000), likely provide a competitive advantage over other phytoplankton species. Less is known about the factors that influence the loss rates of *E. huxleyi*. The two primary mechanisms thought to mediate *E. huxleyi* mortality are viral infection and consumption by zooplankton. While microzooplankton are the main consumers of marine phytoplankton, consuming on average, 60-70% of the daily primary production (Calbet and Landry 2004), grazing pressure on phytoplankton communities dominate by *E. huxleyi* generally appears to be low (Fileman et al. 2002, Olson and Strom 2002), perhaps opening up windows of opportunity for virus-derived mortality in this species (Vardi et al. 2012; Lehahn et al. 2014). Indeed, small shifts in predation pressure can significantly shift phytoplankton population abundance and community composition, and can ultimately influence biogeochemical cycling and the flow of energy throughout the marine planktonic food web (Sherr and Sherr 1988; Strom 2008; Caron et al. 2012).

Protistan predators utilize a variety of sensory mechanisms to identify suitable prey and can be highly selective (Strom and Loukos 1998; Montagnes et al. 2008; Roberts et al. 2011). Both predator grazing rate, as well as its prey preference, are dependent on a range of factors including, size, shape, chemical composition, behavior, and nutritional quality of the alga (Tillmann 2004). Identifying the traits that influence both grazing encounters (searching to

ingestion), as well as prey assimilation and predator growth, will provide an enhanced mechanistic understanding of the relationship between predator and prey. Investigations into heterotrophic protist predation on *E. huxleyi* have primarily focused on the role that DMSP/DMS production plays in mediating grazing interactions. While it has been observed that DMS can attract potential predators (Seymour et al. 2008), in both field and laboratory experiments there is evidence that some predators will select against cells with high cellular DMSP content, preferring low DMSP algae (Archer et al. 2001; Strom et al. 2003; Olson and Strom 2002). However, given the complexity of cell-cell chemical signaling in the marine environment, a debate remains on the mechanisms involved in grazer deterrence by DMSP (Breckles et al. 2010; Strom and Fredrickson 2010).

While the ecological role(s) of coccoliths is still undetermined, there is some suggestion that they may provide protection against predators (Young 1994). Yet, surprisingly little attention has been paid to the role of calcification in modulating interactions between microzooplankton and *E. huxleyi*. Copepods have been shown to consume calcified *E. huxleyi*, however, there is some evidence to suggest that *E. huxleyi* is not a preferred food source (Nejstgaard et al. 1994). In contrast, Hansen et al. (1996) found that *O. marina* preferentially preyed on calcified *E. huxleyi* cells, relative to naked *E. huxleyi* cells and other non-calcified phytoplankton species. The authors attributed this to size selectivity, that *O. marina* may prefer the larger, calcified cell. This observation seems to conflict with several field studies, where heterotrophic protist grazing rates on *E. huxleyi* were found to be lower than other members of the phytoplankton community (Fileman et al. 2002; Olson and Strom 2002; Merico et al. 2004). Further, this depressed grazing pressure has been hypothesized to play an important role in *E. huxleyi* bloom formation and persistence. Therefore, understanding predator grazing and growth kinetics based on calcification is key for predicting both *E. huxleyi* abundance and its impact on biogeochemical cycling.

In order to examine the role of calcification on grazing interactions, five *E. huxleyi* strains were chosen as prey for grazing experiments, based on their basal particulate inorganic carbon (PIC) concentration (Table 1). In order to minimize DMSP as a potential factor impacting predator-prey interactions, we primarily used *O. marina* as a predator. Additionally, we also observed grazing interactions with two additional predators, *Gyrodinium dominans* and *Protoperidinium* sp. These predators have been shown to be the dominant heterotrophic

dinoflagellates during an *E. huxleyi* bloom (Olson and Strom 2002). The utilization of multiple predators allowed us to obtain a more comprehensive understanding of the role of calcification on grazing interactions.

METHODS

General culturing procedures – Five strains of *Emiliania huxleyi* (National Center for Marine Algae: CCMP373, CCMP374, CCMP379 and Plymouth Algal Culture Collection: DHB607, DHB624; Table 1) were used in these experiments. *Isochrysis galbana* (CCMP1323) was used to rear the predators prior to grazing experiments. All phytoplankton cultures were grown in 0.2-µm sterile-filtered autoclaved seawater (FSW), enriched with f/2 –Si media (Guillard 1975). The predators, *Oxyrrhis marina* (LB1974; UTEX Culture Collection), *Gyrodinium dominans* (SPMC 103; Shannon Point Marine Center culture collection), and *Protoperidinium* sp. (PRL2; from S. Menden-Deuer) were cultured in FSW only. All cultures were maintained on a 12:12 h light:dark cycle at 18°C, salinity of approximately 30, and a light intensity of 85-100 µmol photon m^{-2} s^{-1} for the phytoplankton cultures, and 20-30 µmol photon m^{-2} s^{-1} for *O. marina*. The cultures were not axenic. Phytoplankton cultures were transferred every 7-10 days to maintain exponential growth. Prior to the grazing experiments, stocks of each predator were maintained on *I. galbana* (50,000 cells mL^{-1} final concentration), fed twice weekly. Unless otherwise specified, cell concentrations of both predator and prey cultures were determined by microscope counts using samples fixed with 1% Lugol's solution. Hereafter, all phytoplankton strains will be referred to by their strain number.

Experimental set-up – The functional grazing response of *O. marina* to five strains of *E. huxelyi* was measured by calculating the change in prey abundance over 48 h, at five initial prey concentrations (5000 – 80000 cells mL^{-1}; 70 - 1080 ng C mL^{-1}; see Table 2) for each strain examined. Initial predator concentration was approximately 200 cells mL^{-1}. Seawater-, predator-, and prey-only controls were also monitored. Organisms were first combined in 2 L cultures at the appropriate concentrations, mixed gently, then triplicate aliquots were gently poured into 250 mL clear polycarbonate bottles. Sample bottles were placed randomly on a plankton wheel with a rotation of 1 rpm, and maintained on a 12:12 h light:dark cycle at 18°C, and light intensity of 20-30 µmol photon m^{-2} s^{-1} for 48 h. The 2 L bottle was sampled for initial measurements and the polycarbonate bottles after 48 h. Samples were taken for abundance, particulate organic and inorganic carbon, and sulfur measurements.

Calcified (374) and non-calcified (624) strain of *E. huxleyi* were used to measure ingestion rate and predator growth rate of *G. dominans* and *Protoperidinium* sp. Organisms were

combined in triplicate 40 mL tissue culture flasks with the final concentration of prey and predators at 30,000 and 200 cells ml^{-1} respectively. Predator- and prey-only controls were also monitored. The tissue culture flasks were incubated under the same conditions as listed above. Samples were taken after 24 h for *E. huxleyi* and predator abundance only.

Abundance – To calculate *E. huxleyi* abundance, 200 µL aliquots were taken from each replicate and pipetted into a 96-well plate and run on a flow cytometer (Guava, Millipore). Prior to the experiment, the optimal flow cytometer settings for *E. huxleyi* were determined based on chlorophyll a (692 nm) fluorescence and side scatter (SSC). To enumerate the predators, 5 mL aliquots were preserved using 1% Lugol's solution and enumerated using a Sedgwick Rafter and a ZEISS light microscope.

Cell Biovolume – For each strain, the length and width of 100 live cells were measured using a ZEISS light microscope equipped with image capture and image analysis software (ImageJ). Cells were measured live to avoid changes in cell size due to preservatives. Cell volume was determined assuming the shape of the cells were spheres.

Organic and Inorganic carbon – Unless otherwise noted, particulate inorganic carbon (PIC) was determined by calculating the difference between measured total carbon (TC) and particulate organic carbon (POC) values. A total of 80 - 140 mL was filtered over two pre-combusted (450ºC, 24 h), glass fiber filters with the volume evenly split between the two filters. Filters were then placed into pre-combusted glass scintillation vials and dried in an oven for 8 h at 60ºC. For POC measurements, one scintillation vial per replicate was exposed to concentrated HCl fumes (generated from petri dish of 12 N hydrochloric acid) in a glass desiccator for 24 h in order to drive off the PIC. Filters were then dried for 4 h at 60ºC to eliminate any remaining moisture. All scintillation vials were kept in a desiccator until they were pelleted in tin foil discs and analyzed with an EA1112 CHN analyzer. Only samples from the calcified *E. huxleyi* strains were examined using this method. For strains 373, 374, and 379 values reported in the table are taken from Strom et al. 2003a.

Ingestion efficiency - Differences in cell sizes can influence encounter rates between predators and prey, and therefore influence ingestion rates. Calculation of ingestion efficiency (ingestion rate divided by encounter rate) can provide an understanding of how efficient *O. marina* was at ingesting the cells it intercepted, irrespective of differences in encounter rate due to differences in prey cell sizes. To calculate an ingestion efficiency, calculations were made to gain a basic estimate of the volume swept clear as a function of detection radius and swimming speed, using the model of Gerritsen and Strickler (1977):

$$Z = R^2\pi/3((u^2 + 3v^2)/v) \times C$$

R is the distance at which a predator can detect prey. For direct interception feeders, we used an R of the sum of the radii for both the prey and prey cells, following the approaches advanced in Shimeta and Jumars (1991) and Kiørboe (2008), u is the mean prey swimming speed (which is negligible for all strains), v the predator swimming speed (with v > u), and C the prey concentration. Z was calculated for each strain and each prey concentration separately, and has the units cells predator^{-1} d^{-1}. The predator swimming speed was kept constant at 350 µm s^{-1} (Menden-Deuer and Grünbaum, 2006). The ratio of ingestion rate to encounter rate was then calculated.

Model - To quantify the ramifications of the observed ingestion and resultant predator growth rates on a larger, ecosystem level, we formulated an individual-based growth model that predicted *E. huxleyi* change in abundance over time at bloom concentrations. A seed concentration of 5 x10^5 *E. huxleyi* cells L^{-1} and 50 *O. marina* cells L^{-1} was used in combination with the empirically measured predator ingestion and growth rates from the highest prey abundance treatment was used to predict *E. huxleyi* and predator population changes over 72 hours. This starting concentration of *E. huxleyi* is lower than typical bloom conditions (Tyrrell and Merico, 2004).

Sulfur analyses – In order to confirm that sulfur chemistry did not play a large role in mediating the grazing interactions in the system, we measured DMS and particulate DMSP (DMSP$_p$) Gas chromatography (GC) was used to measure the concentration of dissolved DMSP (DMSP$_d$), DMSP$_p$, and DMS. A Shimadzu GC-14 chromatograph equipped with a flame photometric detector was used for all measurements. The column packing was a Chromosil 330 (Supelco),

operated isothermally at 60ºC. Helium was used as the carrier gas. Due to the volatile nature of the compounds, and the necessity to analyze samples shortly after collection, DMS and $DMSP_d$ were measured initially and after 24 h from experiments with the same prey concentration as treatment 3 (and controls) for all strains. $DMSP_p$ was measured initially and after 48 h for all concentrations and strains examined.

For $DMSP_d$ and $DMSP_p$ measurements, 40 mL aliquots were gently vacuum-filtered (less than 5 mm Hg) through a 0.7 µm pre-combusted glass fiber filter (25 mm diameter) into 50 mL polycarbonate tubes. The filtrate was frozen at -80ºC for $DMSP_d$ analysis. Each filter was placed into a microcentrifuge tube, immediately frozen in liquid nitrogen, and transferred to -80ºC for $DMSP_p$ analysis.

Due to the high volatility of DMS, 3 mL whole-water samples were immediately placed in headspace vials and capped with butyl septa. These samples were immediately read on the GC. $DMSP_d$ samples were thawed at room temperature, and 3 mL aliquots were placed in headspace vials, sparged with nitrogen for 3 min to eliminate any remaining DMS. After sparging, 1 mL of 10 N NaOH was added and the headspace vial was immediately capped. Samples were incubated in the dark at room temperature for 12-24 h. $DMSP_p$ filters were thawed at room temperature and placed in headspace vials with 3 mL of 10 N NaOH, immediately capped and incubated in the dark at room temperature for 24 h.

For all measurements, samples were sparged with nitrogen at 20-30 mL min^{-1} for 2 to 5 min and cryotrapped on liquid nitrogen in Teflon traps, which were then heated with hot water (> 80ºC) and injected onto the column. Nafion dryer tubes were used to remove water vapor. Standards were prepared at different ranges for each type of sulfur measurement based on concentrations measured in preliminary experiments. Standards were prepared in deionized water and processed in the same way as $DMSP_d$ samples. Detection limit of the instrument was approximately 0.1 nM DMS in a 5 mL sample.

Statistics – Grazing and ingestion rate measurements were calculated using equations from Frost (1972) as modified by Heinbokel (1978) to account for the growth of *O. marina* over the sampling period. Also calculated was the percent gross growth efficiency (% GGE = predator growth rate/ingestion rate) is the fraction of prey carbon consumed that is converted to growth. Significant differences in grazing and growth rates, as well as %GGE among strains of *E. huxleyi*

were determined by using a two-way analysis of variance (ANOVA) and Tukey's HSD post-hoc analysis. To compare the ingestion and growth rate data from experiments with *G. dominans* and *Protoperidinium* sp., a one-way ANOVA was used to compare rate differences between the naked and calcified strain. All statistical analysis were performed using MatLAB (v. 8.3) and the significance level was $p < 0.05$.

RESULTS

Consumption rates

The ingestion rate (IR) of *O. marina* was significantly impacted by both prey concentration ($p < 0.001$) and by *E. huxleyi* strain (Fig. 1A; $p < 0.001$). Averaged over all prey concentrations examined, ingestion rate was 20% higher in the calcified strains (706 ± 515 pg C pred.$^{-1}$ d^{-1}) relative to naked strains (554 ± 131 pg C pred.$^{-1}$ d^{-1}). However, there was a high level of variability observed in IR between naked and calcified strains, which were driven by differences among the strains. Among strains, average ingestion rate (IR) ranged from 342 ± 141 pg C pred.$^{-1}$ d^{-1} in strain 607 to 1070 ± 205 pg C pred.$^{-1}$ d^{-1} in strain 624. The average IR of strains 374, 379, and 607 were not significantly different from one another, but they were significantly lower (63%; $p < 0.001$) than the average IR of strains 373 and 624. Further there was a significant interaction ($p = 0.003$) between prey concentration and *E. huxleyi* strain, indicating that changes in IR based on prey concentration were strain-specific. For all strains, except 607, IR increased as prey availability increased. In the case of 607, the highest IR observed was in treatment 3 (500 ± 24 pg C pred.$^{-1}$ d^{-1}; Table 2), 30% greater than the IR observed when the most prey was offered (347 ± 17 pg C pred.$^{-1}$ d^{-1}).

Similar to IR, the observed clearance rate (F) was highly strain dependent and significantly different both among strains ($p < 0.001$) and across prey concentrations (Fig. 1B; $p < 0.001$). Further, there was a significant interaction between prey concentration and clearance rate ($p = 0.01$) indicating that the changes in clearance rate observed, as prey concentration increased was significantly different based on strain. When averaged across prey concentration, the average F_{avg} of *O. marina* was not significantly different between naked (1.3 ± 0.6 µL pred^{-1} d^{-1}) and calcified (1.9 ± 0.6 µL pred^{-1} d^{-1}) strains. The lowest F was observed on strain 374 (0.79 ± 0.4 µL pred^{-1} d^{-1}), while the highest observed in strain 624 (2.5 ± 0.6 µL pred^{-1} d^{-1}). The F on

strain 374 was consistent across all prey concentrations examined. In the remaining strains, F decreased as prey concentration increased. In strain 607 this decrease was the most dramatic, with an F of 1.7 ± 0.8 µl $pred^{-1}$ d^{-1} at the lowest prey concentration, compared to 0.32 ± 0.5 µL $pred^{-1}$ d^{-1} at the highest. For strains 624, 373, and 379 F decreased 40-50% from the lowest to highest prey concentration.

The overall volume swept clear calculated for all strains ranged from 0.83 – 1.0 µl $pred^{-1}$ d^{-1}. Given the small difference in the volume swept clear in the encounter rates, the calculated ingestion efficiency was driven by the observed differences in IR. The ingestion efficiency (IE), defined as the fraction of those cells ingested when encountered, was significantly different between all strains examined ($p = 0.004$), as well as prey abundance (Fig. 2; $p = 0.02$). Strain 607 had the lowest average IE (19 ± 10 %) and strain 373 having the highest (49 ± 13 %). Further, in all strains examined, IE decreased between 31 – 82% with increasing prey concentration.

In order to more broadly assess the impact of calcification on grazing by heterotrophic dinoflagellates, more limited experiments using one strain each of calcified and naked *E. huxleyi* were conducted with the thecate heterotrophic dinoflagellate *Protoperidinium* sp. and the naked dinoflagellate *G. dominans*. In both species, IR was significantly different between calcified and non-calcified strains of *E. huxleyi* (Table 3). The ingestion rate of *G. dominans* on strain 624 was reduced by 29 ± 4% relative to strain 374 ($p = 0.02$). The difference in ingestion rate of *Protoperidinium* sp. between a calcified and non-calcified strain was even more extreme, with the predator only consuming 0.41 ± 0.05 pg C $pred^{-1}$ d^{-1} of strain 624 compared to 0.41 ± 0.05 pg C $pred^{-1}$ d^{-1} when fed strain 374 ($p = 0.003$).

Predator growth rates (µ)

The growth rate (µ) of *O. marina* was also significantly different based on prey concentration ($p = 0.001$) and these differences were strain-specific (Fig. 3A; $p = 0.004$). Furthermore, on average, µ of *O. marina* was 0.44 ± 0.2 d^{-1} on non-calcified strains, and 0.15 ± 0.1 d^{-1}, or 66% lower, on calcified strains. This difference between calcified and non-calcified strains was most pronounced at the higher prey concentrations. In non-calcified strains, maximum growth ($\mu_{\square\square\square}$) of *O. marina* was observed at the two highest prey concentrations, 59% higher than the growth observed at the lower prey concentrations. Conversely, in calcified

strains the trend was the opposite with μ□□□found at the lowest prey concentrations, 95% greater than observed at the highest prey concentrations.

The significantly different μ and grazing rates of *O. marina*, translated to significant differences in growth gross efficiency (GGE) among the strains (Fig. 3B; $p < 0.001$). On average, grazing on strain 607 resulted in a negligible GGE, whereas 374 resulted in the highest GGE at 67 ± 4%. There were also strain-specific differences in GGE as a function of prey concentration. For calcified strains GGE was highest at the lowest prey concentrations, and decreased 70 – 100% at the higher prey concentrations. Conversely, in the naked strains, the lowest GGE was found at the lowest two prey concentrations, and increased 70-80% in the two highest prey concentrations.

Feeding on calcified and non-calcified *E. huxleyi* resulted in significantly different μ for both *G. dominans* and *Protoperidinium* sp. (Table 3). *Gyrodinium dominans* grew 35 ± 4% faster when consuming non-calcified *E. huxleyi* relative to the calcified strain 624 ($p = 0.02$). *Protoperidinium* sp. grew poorly on all strains of *E. huxleyi* examined, however μ □□□□□□□□□□□□□□□□□□□□□□□□□□□□□0.03 ± 0.01 compared to 0.11 ± 0.02 when feeding on strain 374 ($p = 0.006$).

Population-level ramifications

The change in abundance of a modeled *E. huxleyi* population was significantly strain-specific ($p = 0.01$; Fig. 4A). Over 72 hours, all three naked strains were consumed completely, whereas the abundance of calcified strains only decreased between 11 ± 4 – 66 ± 10 %. This was the direct result of the impact that consuming either calcified or naked strains had on predator μ. Changes in predator abundance over time were strain-specific ($p = 0.02$; Fig. 4B). Predator abundance grew an order of magnitude greater in strains 373, 374, and 379 during the same time period, whereas in strain 624, abundance increased slightly. In strain 607, predator abundance decreased slightly over 72 hours, due to the negative μ of the predator when ingesting this strain.

Sulfur chemistry

There were observable differences in the DMS and $DMSP_d$ concentrations in the presence of the predator (Table 4). When the predator was absent, DMS and $DMSP_d$ were below the detection limit of the instrument. In the presence of the predator, DMS and $DMSP_d$

concentrations were measurable, but only for strains that had high initial intracellular $DMSP_p$ concentrations (Table 1), 607 and 379. Between both strains, DMS concentration ranged from 7.5-9.3 µM, and $DMSP_d$ concentration between 1.1-3.6 µM. The magnitude of increase in concentration in DMS and $DMSP_d$ in the presence and absence of the predator was not significantly different between strains 607 and 379. The measured DMS and DMSPp did not follow any of the patterns observed in the ingestion or digestion rate data.

DISCUSSION

Prey size is generally considered the most important first-order determinant of whether a prey cell is ingested by a predator. However, intrinsic behavioral, chemical, and morphological cellular traits are also important in dictating selection by a predator (Tillmann 2004). In order to understand the species-specific impacts of microzooplankton on phytoplankton at both the cellular and population level, traits that deter or enhance predation need to be identified. Our results demonstrate that calcification strongly dictates long-term success of both *E. huxleyi* and the population abundance of heterotrophic protists.

We measured ingestion rates (IR) of both naked and calcified *E. huxleyi* strains, by three heterotrophic protist predators. IR ranged from 0.4 – 2336 pg C $pred^{-1}$ d^{-1} (0.6 – 250 cells $pred^{-1}$ d^{-1}), and were dependent on initial prey concentration and strain. Only one other study has previously measured grazing on calcified *E. huxleyi* (Hansen et al. 1996), albeit a different strain then used here, and our measured IRs were slightly higher (~20%). Further, our observed IRs on naked strains were similar to previous rates published for the same strains (Strom et al. 2003a). Using the data from the *O. marina* grazing experiments, we also calculated ingestion efficiency (IE), in order to take into account how encounter rate can change with different prey biovolumes, and how that may impact grazing outcomes. We found no differences in IE between the strains examined, indicating that the measured IR and clearance rates (F) are not being driven by differences in cell size. There are many additional factors, unrelated to size, that could influence IR, including chemical composition of the prey item (Bergkvist et al. 2008).

Both DMS and DMSP have been implicated as infochemicals that may influence grazing interactions between microzooplankton predators and phytoplankton prey. In these experiments, an increase in DMS and $DMSP_d$ concentration was observed in the presence of the predator in treatments where strains had a high initial cellular DMSP concentration, reinforcing the idea that grazing can increase DMS concentrations in the water column (Wolfe et al. 1994; Wolfe and Steinke 1996; Wolfe et al. 2000; Evans et al. 2007). These experiments were not axenic, so it is hard to determine the effect of bacteria to the overall DMS concentration, however treatment blanks did not show significant production of DMS by bacteria. Regardless, increases in DMS did not significantly influence the grazing success of *O. marina*. This is similar to previous studies, where *O. marina* readily ingested both low and high DMS-producing strains (Wolfe and Steinke 1996; Strom et al. 2003a). Further, bulk additions of DMS have been shown to not

362 inhibit feeding by *O. marina* (Strom et al. 2003b). In order to eliminate DMSP as a factor, in
363 experiments with *G. dominans* and *Protoperidinium* spp., the *E. huxleyi* strains used had similar
364 intracellular DMSP concentrations. Thus, DMS/DMSP did not play a major roll in influencing
365 ingestion success for the predators in these current experiments, opening the door for other
366 physiological and chemical traits that influence the variability in ingestion rates observed.
367 Montagnes et al. (2008) describes six steps in protistan prey capture: searching, contact,
368 capture, processing, ingestion, and digestion. In order to understand the role of calcification on
369 the overall consumption success of microzooplankton, each step in the predation process should
370 be considered. We observed predator-specific responses in ingestion on calcified verses non-
371 calcified strains. Our results demonstrated that for *O. marina,* calcification in *E. huxleyi* provided
372 no impediment to ingestion, despite producing negative consequences. For the two other
373 heterotrophic protists examined, *Protoperidinium* sp. and *G. dominans* ingested the calcified
374 strain at a significantly lower rate than the naked strain. Both, *G. dominans* and *Protoperidinium*
375 spp. have been shown to ingest a range of phylogenetically diverse phytoplankton species (Jeong
376 and Latz 1994; Jeong et al. 2004; Kim and Jeong 2004; Schmoker et al. 2011), and *E. huxleyi*
377 would be in the range of prey size that could be consumed by these predators. These results
378 support the notion that grazing interactions in the plankton are highly species-specific, and that
379 while some predators readily consume specific prey-types, others are more selective. Notably,
380 we did not observe ingestion trends as a function of ploidy; strain 379, which was predominantly
381 naked, haploid phase cells, was included in experiments to test for this possibility. Kolb and
382 Strom (2013) found a significant reduction in IR of haploid *E. huxleyi* relative to diploid cells,
383 and suggested that the haploid may have an inducible anti-predatory defense against
384 microzooplankton predators. This observation could be specific to the ciliate predator used in
385 their study, as well as the experimental method employed by Kolb and Strom (2013), which was
386 a short-term (30 minute) observation of prey uptake (i.e. food-vacuole method). Furthermore,
387 such differences in grazing on haploid and diploid cells could also be due to other strain-specific
388 factors, but in these experiments had no bearing on *O. marina* ingestion rate. These results are
389 perhaps not surprising, considering *O. marina* is a well-known generalist predator of other
390 protists, bacteria, and even cohorts (Davidson et al. 1995).
391 When grazer μ was assessed across strains of *E. huxleyi*, a much clearer picture emerged.
392 While all predators used in this study were able to search, contact, capture, process, and ingest

393 both naked and calcified *E. huxleyi*, we found they grew at a higher rate, and more efficiently on
394 naked strains compared to the calcified strains. The impact of *E. huxleyi* on the growth rate (μ) of
395 these heterotrophic protists has not been previously reported. For *O. marina*, the μ observed on
396 naked *E. huxleyi* when at high concentrations (0.62 – 0.99 d^{-1}) were similar to those observed for
397 another prymnesiophyte, *Isochrysis galbana* (0.8 – 1.3 d^{-1}; Goldman et al. 1989). Conversely, the
398 μ of *O. marina* on the calcified strains, were approximately half of those observed on the naked
399 strains. For *G. dominans*, it has been observed that nanoplankton are the optimal food source for
400 these predators (Hansen 1992), however, the μ observed in these experiments are lower than
401 reported for *G. dominans* growing on a wide array of phytoplankton species (0.50 – 1.13 d^{-1};
402 Kim and Jeong 2004). In the case of *Protoperidinium* sp., we observed minimal growth on *E.*
403 *huxleyi*. Previous research on three species of *Protoperidinium* demonstrated that while they can
404 survive when cultured with a wide range of food types, only significant measurable growth has
405 been observed when grazing on diatoms (Menden-Deuer et al. 2005). While *E. huxleyi* clearly
406 did not support maximum μ for either *Protoperidinium* sp. or *G. dominans*, the negative effects
407 of calcified prey were clearly evident.
408 Lower μ □□□□□□□□□□□□*Protoperidinium* sp. or *G. dominans* can largely be
409 attributed to their lower IR and therefore nutritional intake. However, in the case of *O. marina*,
410 IRs on at least one calcified strain were the highest observed in this study, while GGE on both
411 calcified *E. huxleyi* strains were lower at high prey concentrations. This suggests that either
412 calcified cells are nutritionally deficient compared to their non-lithed counterparts, or that the
413 digestion of calcified cells is difficult for this predator. Particulate organic carbon was slightly
414 (5%) higher in the calcified strains relative to the non-calcified strains in these experiments;
415 therefore the calcified strains do not appear to be a nutritionally deficient carbon source.
416 Alternatively, the predators may have been unable to assimilate calcified *E. huxleyi*. The
417 heterotrophic protists food vacuole pH is approximately 3 to 5 during the digestion process, and
418 the enzymes associated with protist digestion work optimally under low pH conditions (Nagata
419 and Kirchman 1992; Gonzalez et al. 1993). Hypothetically an influx of lith-derived calcite could
420 buffer the pH in the food vacuole, reducing the ability to conduct digestion. Therefore, the
421 predators may be able to only partially digest calcified *E. huxleyi*, and simply cannot efficiently
422 convert calcified *E. huxleyi* consumption into predator growth. This has been hypothesized to
423 occur in coral reef fish that ingest calcifying algae **(**Lobel 1981; Schupp and Paul 1994).

The benefit of calcification for coccolithophores has remained an open question. It has been proposed that *E. huxleyi* has coccoliths as a mechanism of defense against predation (Young 1994); however there has been no direct support of this hypothesis. Regardless of the specific mechanism, a reduction in predator growth rates as the result of ingesting calcified *E. huxleyi* would result in a positive-feedback loop for the alga. While there may be an initial decrease in the *E. huxleyi* population, our modeling results using empirical experimental data show that the population abundance for the heterotrophic protists feeding on calcified *E. huxleyi* will either decrease over time or fail to keep pace with a growing *E. huxleyi* population. Ultimately, this will result in enhanced *E. huxleyi* survival relative to naked strains, and greater potential for population growth. Therefore, in this respect, coccoliths do convey a protection mechanism for *E. huxleyi* populations. In an ecological sense, this advantage could be analogous to the potential benefits of diatom-derived polyunsaturated aldehydes released from wounded cells, which result in the inhibition of copepod reproduction (Miralto et al. 1999; Ianora et al. 2004). In both cases the trait results in indirect defense for prey populations by disrupting the reproduction of their grazers. While it is not possible to generalize that our results will apply for all microzooplankton grazers, they do help explain observations of lower grazing pressure on *E. huxleyi* relative to other phytoplankton species (Fileman et al. 2002; Olson and Strom 2002; Merico et al. 2004). These results also leave the door open for other loss processes, such as infection by the *E. huxleyi* virus, as being important mortality mechanisms in natural populations.

Conclusions

Species interactions are among the main factors that shape planktonic communities. Our results demonstrate that microzooplankton-phytoplankton interactions can be complex, and influenced by strain-specific factors. The experiments shown here highlight the wide variability in functional grazing response of heterotrophic dinoflagellate predators to five strains of the same prey species (*E. huxleyi*) and suggest that parameters other than cell size (i.e. calcification) are crucial in influencing host-grazer interactions. Our results demonstrate that while calcification is not significantly influential in mediating ingestion rate for all predators, it has a major impact on the growth of three heterotrophic dinoflagellate species. As demonstrated in our simple model, this effect on predator growth could result in increased survival of this globally important alga, and likely facilitates the formation and/or duration of *E. huxleyi* blooms.

Together, these results also highlight the potential role of calcification as a negative selective force against microzooplankton grazing, thereby facilitating the flow of calcite through the biological pump. The identification of traits that have significant influence on prey selectivity by heterotrophic protists is essential for increased understanding of biogeochemical cycling and transfer of energy and material through the marine food web, but also for better parameterization of both trait-based and ecosystem-based models.

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TABLE LEGEND

Table 1 – Strain number, ploidy (H = haploid; D = diploid), calcification state, diameter (µm), biovolume (μm^3), particulate organic carbon (POC; pgC $cell^{-1}$), particulate inorganic carbon (PIC; pgC $cell^{-1}$), DMSP lyase production ($DMSP_l$; fmol $cell^{-1}$ min^{-1}), and intracellular DMSP ($DMSP_p$; mM) concentration for all five strains examined. Unless noted, all physiology data was determined from these experiments. Average values are shown (at least n = 3 for all measurements), with one standard deviation from the mean in parenthesis.

Table 2 – The concentration of prey and predators used (brackets) and the experimental design for the functional response experiment with strains of *E. huxleyi* exposed to the predator *O. marina*. This experimental design was repeated for all five strains of *E. huxleyi*.

Table 3 – The ingestion (pg C $pred^{-1}$ d^{-1}) and growth rate (d^{-1}) of two heterotrophic protists, *Gyrodinium dominans* and *Protoperidinium* sp. feeding on either a calcified (strain 624) or non-calcified (strain 374) strain of *E. huxleyi*. Average values are shown (n =3), with one standard deviation from the mean in parenthesis.

Table 4 – Mean concentrations (± standard deviation) of DMS (µM) and $DMSP_d$ (µM) in both the presence and absence of the predator. Concentrations that were below the detection limit of the instrument are listed as 'ND'. Concentrations of both DMS and $DMSP_d$ increased in the presence of the predator only in those strains with high initial cellular $DMSP_p$ concentrations (See Table 1). No data was collected on strain 373.

FIGURE LEGEND

Figure 1 – Ingestion rate (pg C pred.$^{-1}$ d^{-1}; A) and clearance rate (µL pred.$^{-1}$ d^{-1}) of *O. marina* on all five strains of *E. huxleyi* examined. While strain-specific differences in ingestion and clearance rate were observed, there was no clear delineation in either rate measurement between calcified (black) and non-calcified (gray) strains. Error bars represent one standard deviation of the mean.

Figure 2 – Ingestion efficiency (%) of *O. marina* on all five *E. huxleyi* strains examined. The ingestion efficiency is the percentage of cells that were ingested based on those that were encountered by *O. marina*. While strain-specific differences are observed, there are no clear differences between calcified (black) or non-calcified (gray) cells. Error bars are one standard deviation of the mean.

Figure 3 – Growth rate (d^{-1}; A) and gross growth efficiency (%; B) of *O. marina* when feeding on *E. huxleyi*. The predator population experiences significantly higher growth rates when feeding on naked (gray) cells relative to calcified (black) cells, at high prey abundances. Error bars are one standard deviation from the mean.

Figure 4 – Model the population abundance of (A) *E. huxleyi* and (B) *O. marina* over time, using the empirically measured parameters in this study. The population abundance of the naked strains (gray) is rapidly eliminated, resulting in a rapid increase in *O. marina* population abundance. Conversely, the low growth of *O. marina* when feeding on calcified strains (black) results in the calcified population being able to maintain a population over time. Error bars are one standard deviation from the mean, and are generally hidden by the symbol.

Table 1

Strain	Ploidy	Liths?	D (µm)	Biovolume (μm^3)	POC (pgC $cell^{-1}$)	PIC (pgC $cell^{-1}$)	$DMSP_l$ (fmol $cell^{-1}$ min^{-1})	$DMSP_p$ (mM)
373	D	No	4.7 (0.1)[1]	54.3 (0.3)	7.9 (0.09)[1]	-	2.1-5.9[1]	unknown
374	D	No	3.9 (0.4)[1]	31.1 (1.2)	8.1 (0.03)[1]	-	0.01-0.08[1]	32.6 (4.1)
379	H	No	4.1 (0.3)[1]	36.1 (1.1)	8.1 (0.03)[1]	-	0.1-3.0[1]	58.3 (3.3)
607	D	Yes	6.8 (0.6)	113.0 (2.7)	9.5 (0.3)	5.2 (0.9)	unknown	46.5 (6.2)
624	D	Yes	5.6 (0.8)	91.9 (2.2)	12.1 (1.3)	4.8 (0.2)	unknown	19.6 (1.6)

[1]data from Strom et al. 2003a.

Table 2

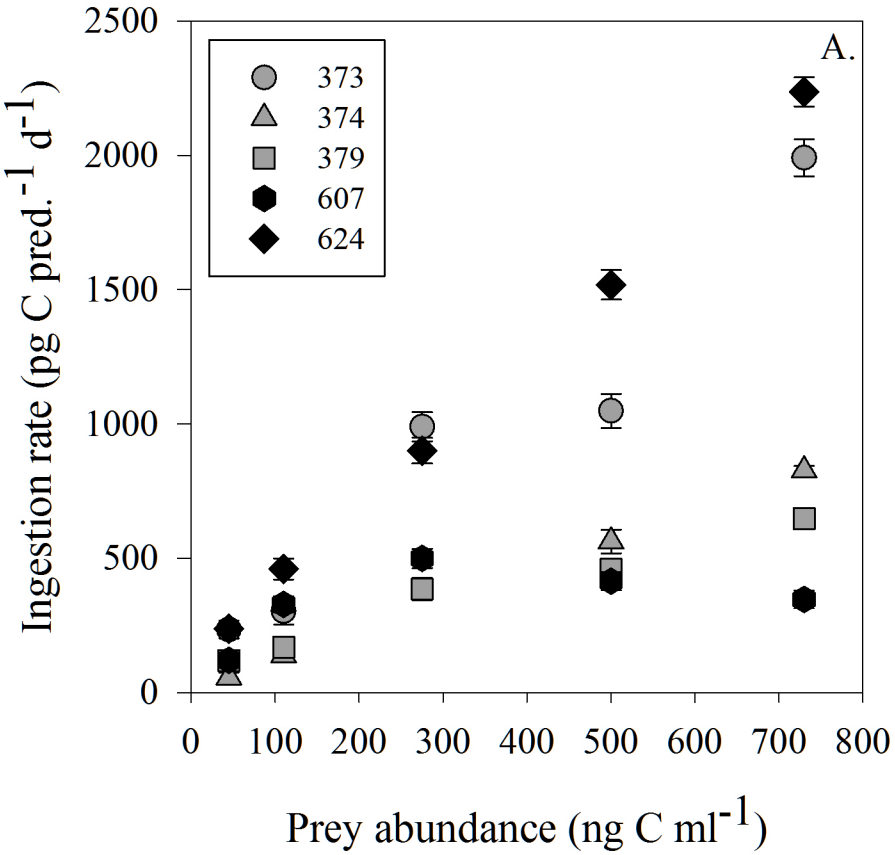
Treatments	[Prey] (cells mL^{-1})	[Prey] (ng C mL^{-1})	[Predator] (cells mL^{-1})	Prey:predator	Replicates
1	5000	70	200	25	3
2	12000	160	200	60	2
3	30000	400	200	150	3
4	55000	750	200	275	2
5	80000	1080	200	400	3
Predator only	0	0	200		3
Blank	0	0	0		1

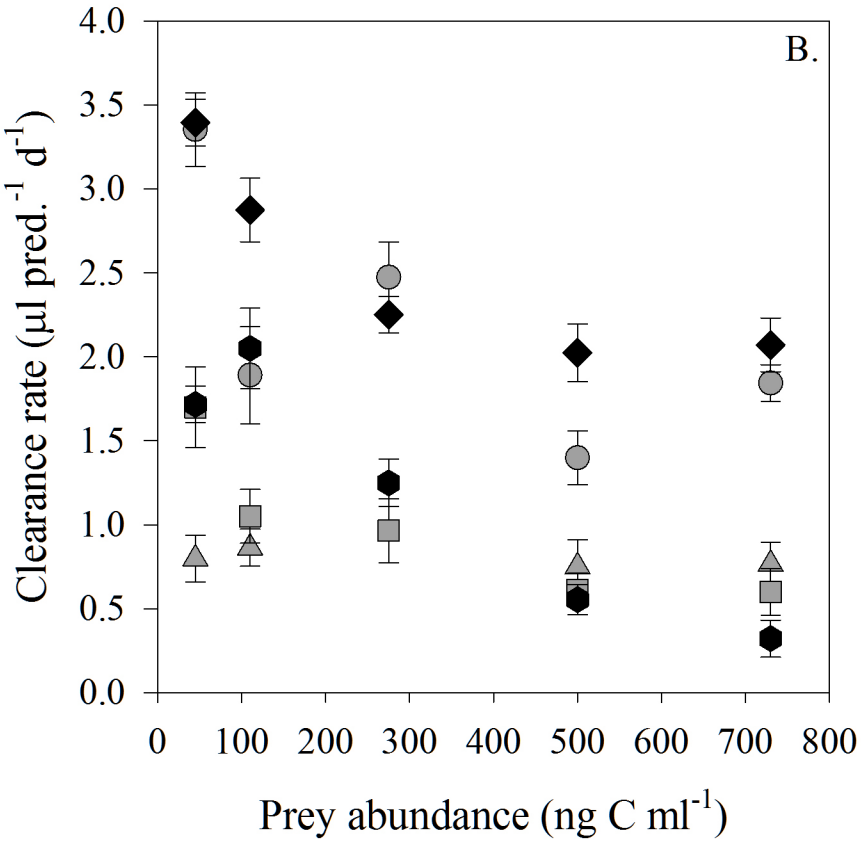
Table 3

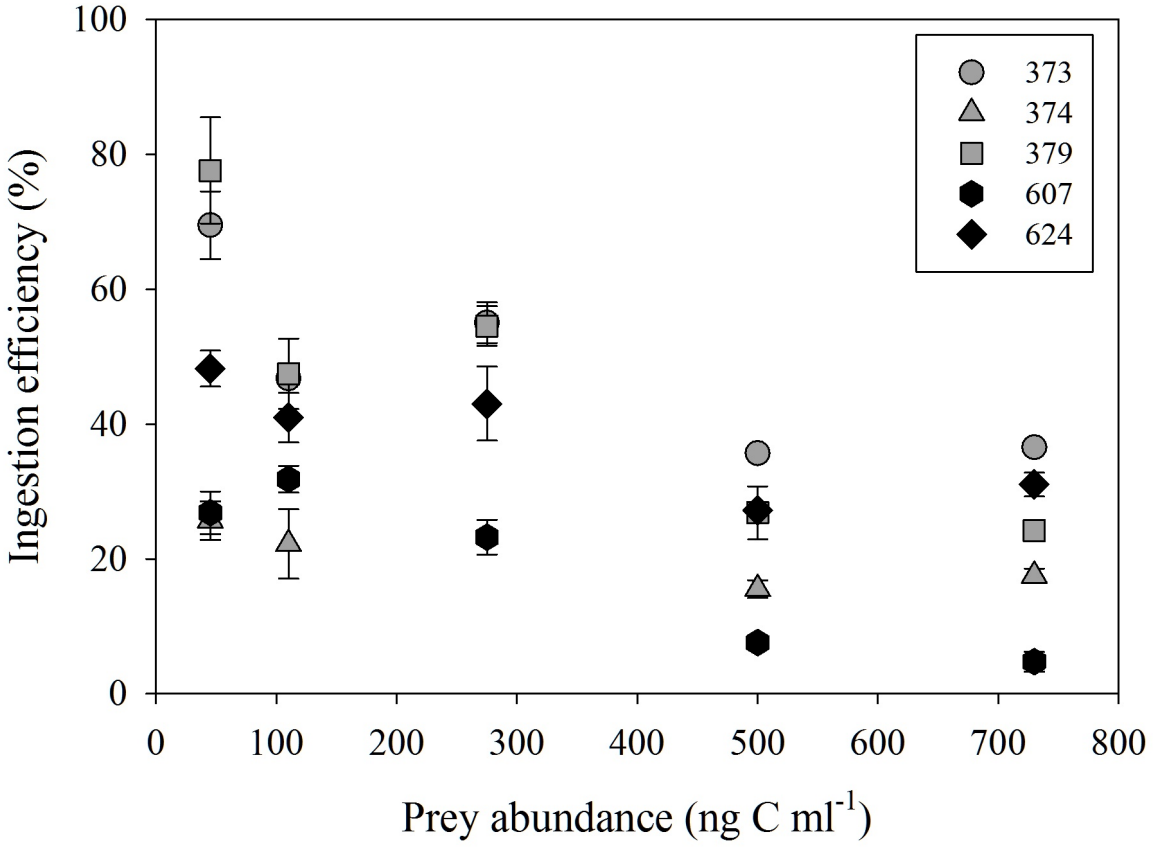
		Ingestion rate (pg C pred^{-1} d^{-1})	Growth rate (d^{-1})
Protoperidinium sp.			
	374	92 (10)	0.11 (0.02)
	624	0.41 (0.05)	0.03 (0.01)
Gyrodinium dominans			
	374	375 (35)	0.23 (0.03)
	624	266 (22)	0.15 (0.02)

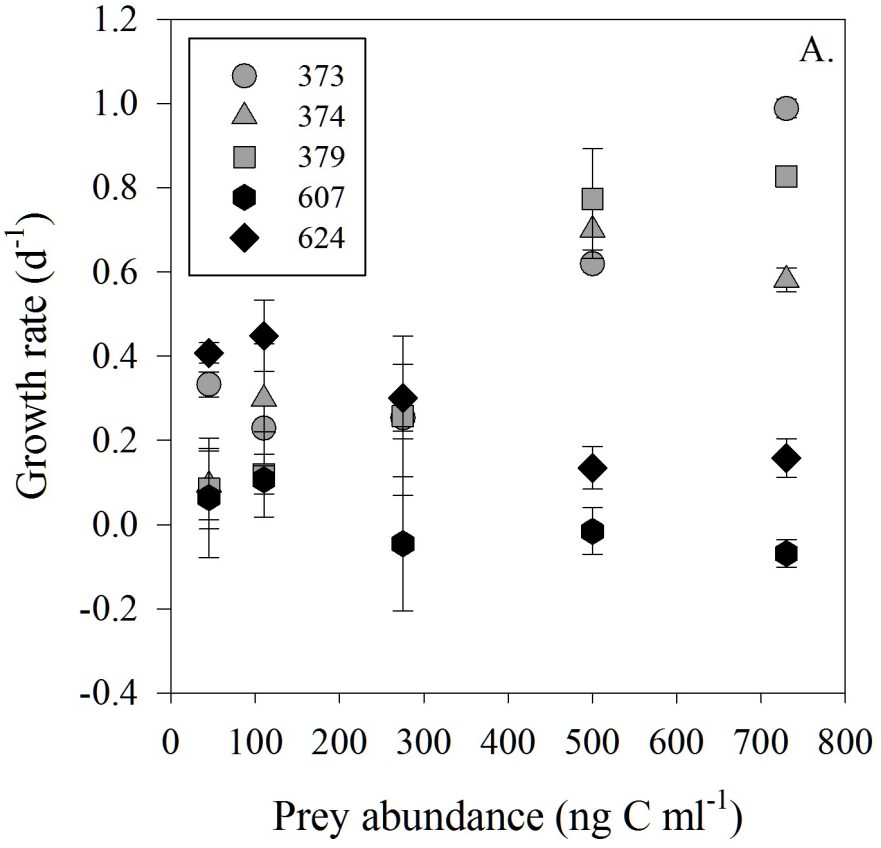
Table 4

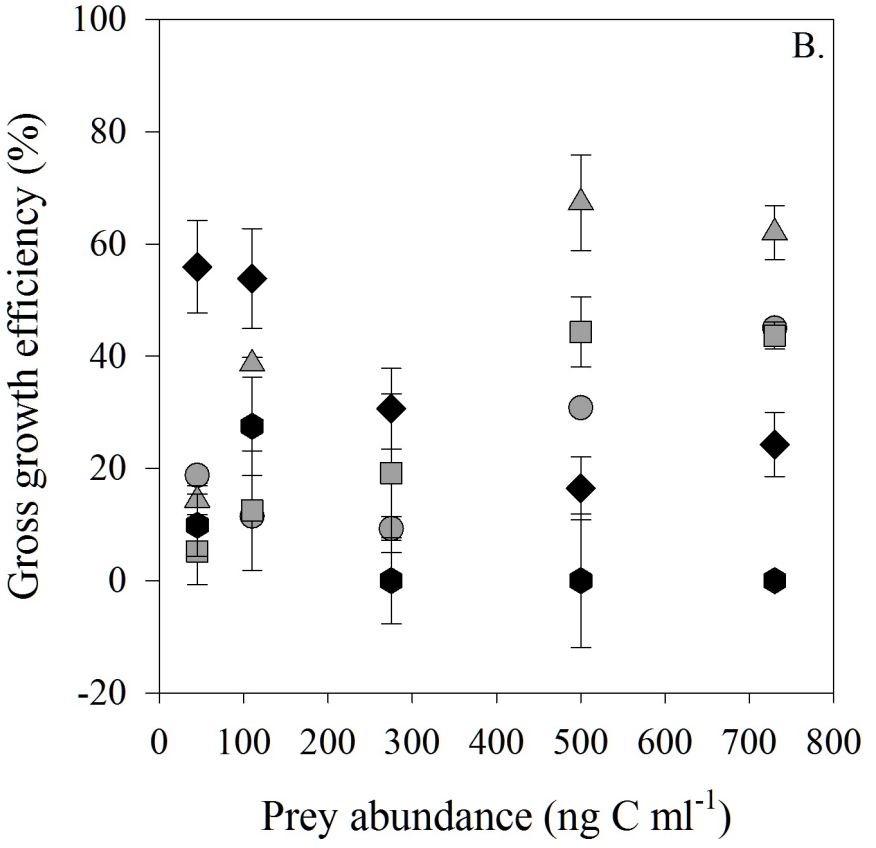
	DMS (μM)		$DMSP_d$ (μM)	
	no predator	+ predator	no predator	+ predator
374	ND	ND	ND	ND
379	ND	7.8 (1.2)	ND	1.2 (1.4)
607	ND	9 (0.2)	ND	3.2 (2)
624	ND	ND	ND	ND

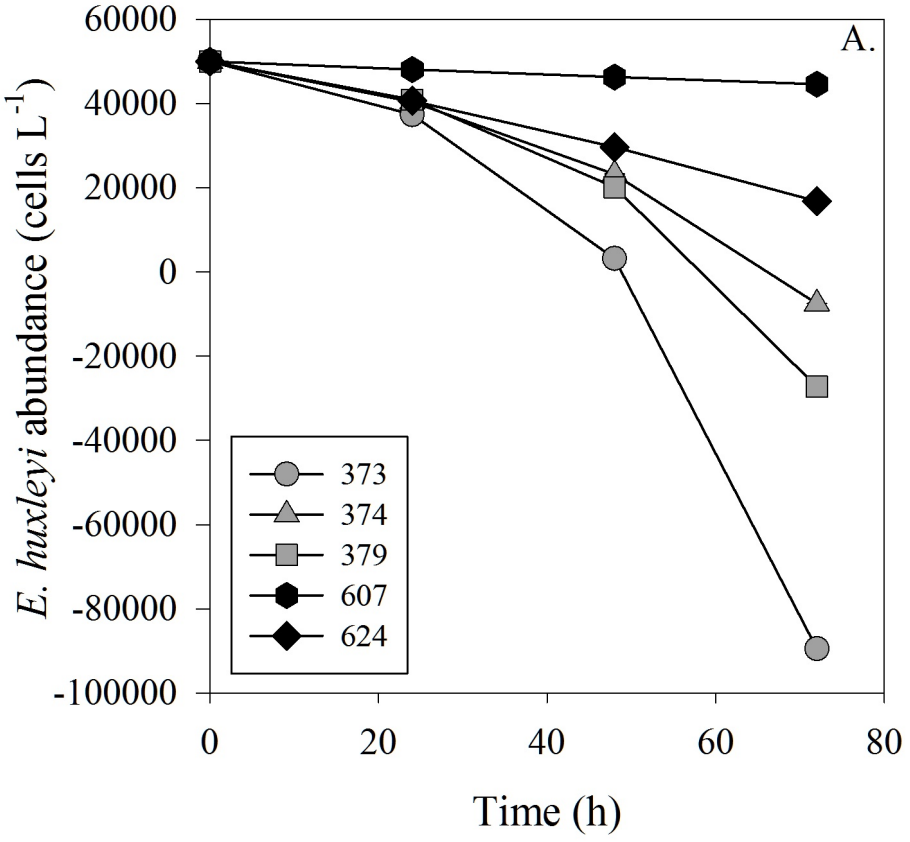

A.
373
374
379
607
624
Ingestion rate (pg C pred.-1 d-1)
Prey abundance (ng C ml-1)
0
500
1000
1500
2000
2500
0
100
200
300
400
500
600
700
800

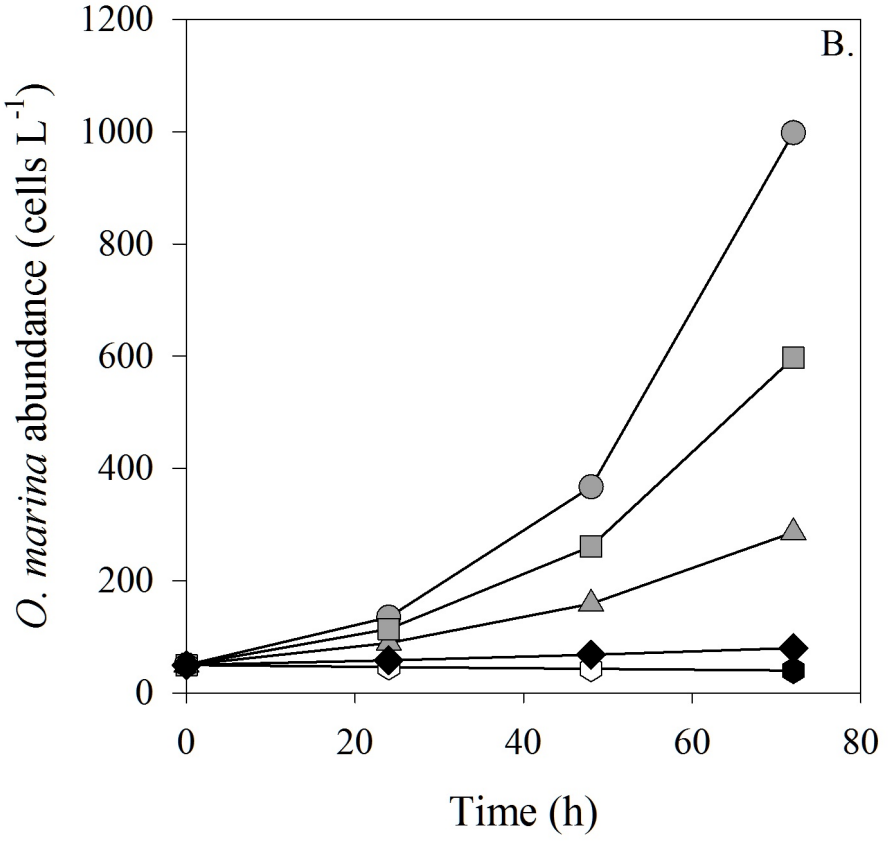

B.
Clearance rate (µl pred.-1 d-1)
Prey abundance (ng C ml-1)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
100
200
300
400
500
600
700
800


Ingestion efficiency (%)
100
80
60
40
20
0
0
100
200
300
400
500
600
700
800
Prey abundance (ng C ml-1)
373
374
379
607
624


A.
373
374
379
607
624
Growth rate (d-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
0
100
200
300
400
500
600
700
800
Prey abundance (ng C ml-1)


B.
Gross growth efficiency (%)
100
80
60
40
20
0
-20
0
100
200
300
400
500
600
700
800
Prey abundance (ng C ml-1)


A.
60000
40000
20000
0
-20000
-40000
-60000
-80000
-100000
E. huxleyi abundance (cells L-1)
0
20
40
60
80
Time (h)
373
374
379
607
624


B.
1200
1000
800
600
400
200
0
O. marina abundance (cells L-1)
0
20
40
60
80
Time (h)